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COMBINED AND SEPARATE ANALYSES OF MORPHOLOGICAL AND MOLECULAR DATA IN THE PLANT FAMILY RUBIACEAE

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Abstract — The Rubiaceae are one of the largest of the families of angiosperms, with over 10 000 species. The tribal and subfamilial classification is provisional due to the lack of phylogenetic hypotheses. The present study of the Rubiaceae is based on 33 genera and three data sets, one morphological and two molecular from chloroplast DNA, restriction sites and *rbc*L sequences. There is much congruence between the morphological and the molecular data sets, but also conflict. For parsimony reasons, the best phylogenetic hypothesis is a tree based on an analysis of the combined data sets. The so-called "total evidence" criterion for the combined analysis is simply a reiteration of the principle of parsimony. In this particular study, the classification would be almost the same even if based on the separate analyses instead of the combined. Despite the inapplicability of consensus trees or trees from separate analyses for phylogenetic hypotheses and classification, separate analyses may provide important information. It is the best way to reveal conflicts between different data sets. Knowledge of the conflicts can promote further detailed investigation in order to improve understanding of characters and phylogenetic hypotheses. In this study, the tribe Vanguerieae provides such an example; morphological data support a position in the subfamily Cinchonoideae, but DNA and a tree based on the combined data support a position in subfamily Ixoroideae. The tribe's position in the morphological tree is probably due to missing information concerning the correct pollen presentation system. Bootstrap fractions and K. Bremer's branch support values are used to evaluate the stability of particular nodes in the trees. Interestingly these values are not always correlated, e.g. in the morphological tree, the node with the highest branch support value has very low bootstrap fraction. The reasons for these differences are unclear, but large differences are presumably more likely to occur on short branches.

Introduction

Until the 1970s, molecular characters were rare in systematic studies but, since then, there has been a tremendous increase in the use of molecular data in various groups of organisms (e.g. humans and african apes, Miyamoto et al. (1987); phyla and kingdoms of prokaryotes, Woese, 1987; angiosperms, Chase et al., 1993). Most systematic journals today contain phylogenetic investigations based on molecular characters alone or in combination with morphological data.

With the appearance of molecular data sets, a debate started about the importance and reliability of molecular versus morphological data (reviews: Patterson, 1987; Hillis, 1987). This debate has continued with methodological issues, such as whether morphological and molecular sets of characters should be analysed separately or together (e.g. Eernisse and Kluge, 1993). As early as 1976, Mickevich and Johnson presented a study of morphological and allozyme data and they analysed the data separately and in a combined analysis. Congruency between the trees from the different data sets were calculated (following the method of Farris, 1973)

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and the distortion (Farris, 1973) of the different trees were compared to a tree from the combined data. In their particular study, Mickevich and Johnson found no difference in reliability between the two sets of characters.

Initially, cladistic classifications were based on consensus trees (Nelson, 1979; Nelson and Platnick, 1981) if different analyses resulted in different fundamental cladograms. However, Miyamoto (1983) argued that phylogenetic relationships must be reconstructed with all available information in a combined analysis. He pointed out (Miyamoto, 1985: 187, 188) that "Consensus cladograms are generated from fundamental cladograms instead of original information. This problem with loss of parsimony becomes important whenever consensus cladograms lose resolution unnecessarily. In contrast, parsimony (Wagner) procedures that work from combined data sets ensure efficient results, because they operate directly on available information", and the conclusion is that "systematists and specialists of particular groups should base their phylogenetic syntheses and general classification on cladograms constructed from combined data sets of available information (Mickevich and Johnson, 1976; Miyamoto, 1983, 1984)". However, Miyamoto also performed separate analyses and stated "Furthermore, fundamental cladograms may be compared by consensus techniques to reveal which clades of general cladograms are stable, unstable, and ambiguous" . . . "Fundamental cladograms supplement general cladograms, and therefore, are useful to phylogenetic synthesis".

Despite these clarifications by Miyamoto (1983, 1985), the debate continued (e.g. Hillis, 1987; Kluge, 1989; Sytsma, 1990; Barrett et al., 1991, 1993; Bull et al., 1993; de Queiroz, 1993; Kluge and Wolf, 1993; Nelson, 1993) and as late as 1994 a symposium at the Willi Hennig Society meeting was dedicated to "Molecular and morphological systematics—total evidence in phylogenetic reconstruction?" Today, most systematists agree that a combined analysis is the most appropriate method for phylogenetic reconstruction and classification, but see no conflict in analyzing the different data sets separately as well.

With separate analyses it is possible to identify incongruences between data sets. Such incongruences can promote further investigations and result in re-evaluation of character homologies. In some cases, the differences in phylogenies may be real due to different histories for, e.g. organelle and nuclear trees. Since Miyamoto, few authors have argued for using consensus trees for classification and/or estimating phylogenies (Hillis, 1987; Swofford, 1991). Others, like Kluge (1989), have totally abandoned the consensus approach or analyses of separate data sets for systematic or phylogenetic purposes. Kluge asserts that from the concept of "total evidence" (Carnap, 1950; Hempel, 1965) it follows that an analysis of morphological and molecular data must be a combined analysis. The principle of using the total evidence also applies, of course, to systematics, but it does not mean that parts of the evidence can not be investigated separately, as may be done in a phylogenetic analysis. It is unfortunate that the expression "total evidence analysis" has been used for the combined analysis, as it may give the false impression that "total evidence" is some additional necessary criterion for choosing the most parsimonious solution.

While much effort has been devoted to discussions on how to perform an analysis with molecular and morphological data, very little has been said outside the sphere of cladists about the crucial message, namely, that a phylogenetic analysis

should include all available information. It is still very common to find molecular studies, published in reputable scientific journals, with no consideration or discussion of morphological data, although such data are available in the literature. In summary, if different data sets are available, there is nothing wrong with analysing them separately, but a phylogenetic hypothesis should be based on an analysis of the combined data sets.

Taxonomic background

The issues raised above may be illustrated with the following case study of molecular and morphological investigations of the angiosperm family Rubiaceae. The study is based on two molecular data sets (sequence data from the *rbc*L gene, ribulose-1,5-bisphosphate carboxylase, and restriction site data, both of the chloroplast genome) and one morphological data set.

Some well known members of Rubiaceae are coffee plant and *Cinchona*, the original source of quinine. The family is biologically and morphologically diverse, with many different life forms and reproductive traits. The life forms vary from tiny herbs, epiphytes, lianas, and shrubs to tall trees, and the various kinds of flowers have different pollination systems. There is great variety of fruits and seeds dispersed by different agents, e.g. dry capsules with wind dispersed seeds, dry dehiscent or indehiscent mericarps, or fleshy and animal dispersed berries or drupes.

The Rubiaceae are an easily circumscribed family, but with problematic infrafamilial classification, especially at subfamilial and tribal level (Bremekamp, 1954, 1966; Verdcourt, 1958; Robbrecht, 1988; Bremer and Jansen, 1991). There are several reasons for this situation: (1) the Rubiaceae are a very large family with about 10 000 species and 640 genera; (2) they are mainly tropical and therefore not well collected; (3) relatively few useful morphological characters have been identified; (4) typically single “cardinal” characters were traditionally used for the classifications; and (5) relatively few systematists have been interested in the family (although the first cladogram from the family was published by Bremer, 1979). Originally the family was split into two subfamilies based on a single character, namely the number of seeds per fruit (cf. Schumann, 1891). This character was totally rejected in the classification schemes that were presented in the middle of this century (Bremekamp, 1954; Verdcourt, 1958). At that time, new subfamilies were described. For one of the former subfamilies a very different circumscription was proposed. Eight (Bremekamp, 1954, 1966) or three (Verdcourt, 1958) subfamilies were accepted, and new cardinal characters were introduced. For example, subfamily Rubioideae was characterized by having raphides (Calcium-oxalate crystals) and albuminous seeds, Ixoroideae by a secondary pollen presentation mechanism and no raphides, and Cinchonoideae by pitted testa cells and absence of raphides. One of the very small subfamilies, Antirheoideae (described as Guettardoideae), including only one tribe, was characterized by the absence of albumin in the seeds, absence of raphides and small cotyledons. The problem with these classifications is that the subfamilies are characterized by single or few characters and some of these have later been shown to be plesiomorphic and shared by the outgroup (Bremer and Struwe, 1992). The latest comprehensive

classification of the family was presented by Robbrecht (1988, 1993a,b), but was not based on any phylogenetic analysis. He accepted four subfamilies (Antirheoideae, Cinchonoideae, Ixoroideae and Rubioideae). Robbrecht enlarged the subfamily Anthireoideae to include all taxa with the character combination "endosperm soft and oily; embryo frequently very large". Since publication of Robbrecht's (1988) classification, two cladistic analyses of Rubiaceae have been published. The analyses were based on restriction site data (Bremer and Jansen, 1991), on morphological data and on a combination of morphological and restriction site data (Bremer and Struwe, 1992). These studies included about five percent of all genera in the family and their results are partially incongruent with one another. Both studies conclude, however, that the wide circumscription of the subfamily Antirheoideae is not supported. Despite these published phylogenies, indicating a different circumscription of the Antirheoideae and also indicating several totally new relationships, Robbrecht's revision discusses but does not implement the results in the classification schemes (Robbrecht, 1993a,b).

Discrepancies between the restriction site data (Bremer and Jansen, 1991) and the morphological data (Bremer and Struwe, 1992) motivated the collection of additional molecular data. Hence, *rbc*L sequences are added here to improve the phylogenetic hypothesis of the Rubiaceae. Furthermore, results from combined versus separate analyses are compared and discussed, including assessment of stability and branch support in the different trees.

Materials and Methods

Thirty-three genera of Rubiaceae representing the four subfamilies and 19 tribes (of 44, fide Robbrecht, 1988) were investigated. Two different molecular data sets from the chloroplast genome were analysed together, one based on restriction site mapping of the chloroplast DNA, including 161 phylogenetically informative characters (Bremer and Jansen, 1991, Table 5) and one on sequence data from the *rbc*L gene including 218 phylogenetically informative characters. The *rbc*L gene has been sequenced from three Rubiaceae genera (Table 1); sequences are accessioned in EMBL as x87145, x87146, and x87147. The remaining 30 sequences included in the analyses have been published previously (Bremer et al., 1995, Table 1).

DNA was extracted, amplified, and sequenced following the same protocols as in Bremer et al. (1995). The *rbc*L data matrices in the phylogenetic analyses comprise characters corresponding to each nucleotide position (27 to 1428, positions 1–26 are excluded as they are identical to one of the primers) of the *rbc*L sequence, but only the 218 phylogenetically informative characters were included in the cladistic analyses.

The morphological character set (Appendix 1) is a corrected and revised version of that analysed by Bremer and Struwe (1992), including 35 characters. Character states for each taxon are found in the data matrix (Appendix 2).

The genus *Galium* (tribe Rubioideae) is our only composite taxon. The morphological and the restriction site data are from *Galium*. It was not possible to sequence the genus, so the *rbc*L sequence of *Rubia tinctorum*, representing a very closely related genus, was used instead.

Table 1
Species investigated for the cpDNA analyses

Species[a]	Voucher information[b]	New *rbc*L sequences reported in this paper will appear in EMBL database under the accession number
Subfamily Antirheoideae		
*Antirhea lucida*GUE	Bremer et al., 1995	
*Cephalanthus occidentalis*CEP	Bremer et al., 1995	
*Chiococca alba*CHI	Olmstead et al., 1993	
*Erithalis fruticosa*CHI	Bremer et al., 1995	
*Exostema caribaeum*CHI*	Bremer et al., 1995	
*Guettarda uruguensis*GUE	Bremer et al., 1995	
*Hintonia latiflora*CHI*[c]	Bremer et al., 1995	
*Vangueria madagascarensis*VAN	Bremer et al., 1995	
Subfamily Cinchonoideae		
*Calycophyllum candidissimum*CIN	Bremer et al., 1995	
*Cinchona succirubra*CIN	Bremer et al., 1995	
*Haldina cordifolia*NAU	Bremer et al., 1995	
*Luculia grandifolia*CIN	Bremer et al., 1995	
*Mussaenda erythrophylla*ISE	Bremer et al., 1995	
*Pinckneya pubens*CON	Bremer et al., 1995	
*Pogonopus speciosus*CON	Bremer et al., 1995	
*Rogiera suffrutescens*RON	Bremer et al., 1995	
Subfamily Ixoroideae		
*Coffea arabica*COF	Bremer et al., 1995	
Enterospermum coriaceum	Bremer et al., 1995	
*Gardenia thunbergia*GAR	Bremer et al., 1995	
*Ixora coccinea*PAV	Bremer et al., 1995	
*Mitriostigma axillare*GAR	Bremer et al., 1995	
Subfamily Rubioideae		
*Coccocypselum hirsutum*COC	Bremer & Jansen, 1991	x87145
*Coprosma pumila*ANT	Bremer & Jansen, 1991	x87146
*Myrmecodia platyrea*PSY	Bremer & Jansen, 1991	x87147
*Hamelia cuprea*HAM	Bremer et al., 1995	
Hoffmannia refulgens ×*ghiesbreghtii*HAM	Bremer et al., 1995	
*Hydnophytum formicarum*PSY	Bremer et al., 1995	
*Nertera granadensis*ANT	Bremer et al., 1995	
*Pentas lanceolata*HED	Bremer et al., 1995	
*Psychotria kirkii bacteriophila*PSY	*Bremer et al., 1995*	
*Rubia tinctorum*RUB[d]	Bremer et al., 1995	
Incertae sedis		
*Boubuardia glaberrima*CIN / HED	Bremer et al., 1995	
*Catesbaea spinosa*CAT	Bremer et al., 1995	

[a]Species names are followed by a three-letter suffix corresponding to the three letters of the tribal names: ANThospermeae, CATesbaeeae, CEPhalantheae, CINchoneae, CHIococceae, COFfeeae, CONdamineeae, GARdenieae, GUEttardeae, HAMelieae, HEDyotideae, ISErtieae, MORindeae, NAUcleeae, PAVetteae, PSYchotrieae, RONdeletieae, RUBieae, VANguerieae. The listed subfamilies of Rubiaceae follow Robbrecht (1988), as do the tribal positions for most taxa; however, * indicates tribal positions according to Bremer (1992).

[b]Reference to literature if the specimen has been used before.

[c]Dr Charlotte Taylor has kindly informed me that this species erroneously was indicated as *Coutarea latiflora* in Bremer and Jansen (1991).

[d]In the molecular analysis the *rbc*L sequence from the tribe Rubieae is from the genus *Rubia* and not from the closely related genus *Galium* which is used in the morphological and restriction site analysis.

Parsimony analyses were conducted using PAUP vers. 3.1.1 (Swofford, 1993) on a PowerMacPC8100/80, with all character changes weighted equally. In cases with polymorphic or uncertain characters, the DNA and the combined data were analysed with uncertain states and the morphological data were analysed with both polymorphic and uncertain states. Only phylogenetically informative characters were included. No duplications of characters between the *rbc*L sequences and the RFLP data set were included. The methods for the searches were heuristic, with random stepwise addition of sequences and 100 replications, and TBR branch swapping with MULPARS on and Steepest descent off. To estimate the support for the particular branches K. Bremer's branch support (B=the extra length needed to lose a branch in a consensus of near-most parsimonious trees, K. Bremer, 1988) and bootstrap fractions (with 1000 replicates; Felsenstein, 1985) were calculated. Tree stability was estimated by the total support (T=the sum of all b values over the tree; Källersjö et al., 1992) and the total support index (TI=T divided by the length of the most parsimonious trees [s]; K. Bremer, 1995).

Results

The morphological analysis —(Fig. 1, Tables 2, 3). When multistate taxa were analysed as polymorphic, 6 equally parsimonious trees resulted (length [S]=179, consistency index [CI]=0.598, retention index [RI]=0.673). The total support (T) value was 36, and the total support index (TI) was 0.201. When multistate taxa were treated as uncertain, the same trees resulted; however, they were much shorter (S=135, CI=0.467, RI=0.673, T=36, TI=0.267). The individual branch support (B) values vary between 1 and 3 steps (in both analyses); hence, consensus trees will be totally collapsed if trees with 4 extra steps are accepted. Only nine of all the nodes have bootstrap fractions above 50% and they are rather low, from 51 to 83%. Three of four subfamilies are recovered.

The molecular analysis —(Fig. 2, Tables 2, 3). The analysis of restriction data and *rbc*L sequences resulted in 13 trees (S=979, CI=0.451, RI=0.714, T=245, TI=0.250). The individual branch support (B) values vary between 1 to 46 steps and to collapse the tree, many (47) extra steps are required. Twenty nodes are supported with bootstrap fractions above 50%, varying between 56 to 100%. As in the morphology analysis the monophyly of three of four families is recovered, but tribal relationships differ. As a result of these conflicts, a strict consensus tree between the two analyses (Fig. 3) is unresolved and largely uninformative.

The phylogenies from the two analyses are similar, however. Both show three groups of taxa more or less corresponding to three of the four subfamilies. The groupings of taxa are very congruent with the tribal positions. There are certain differences, however. Within the subfamily groups there are several differences in how tribes are related. For example, the tribe Hamelieae is closest to Guettardeae in the DNA analysis but closest to the Chiococceae s.l. in the morphological analysis; *Galium/Rubia* of the tribe Rubieae is the sister group to tribe Hedyotideae in the molecular analysis and to tribe Psychotrieae in the morphological analysis. Furthermore, there are two big differences between the two analyses, namely with respect to the positions of the genus *Vangueria*, of the tribe Vanguerieae, and the group of taxa with a semaphyll (*Mussaenda*ISE, *Pogonopus*CON, *Pinckneya*CON,

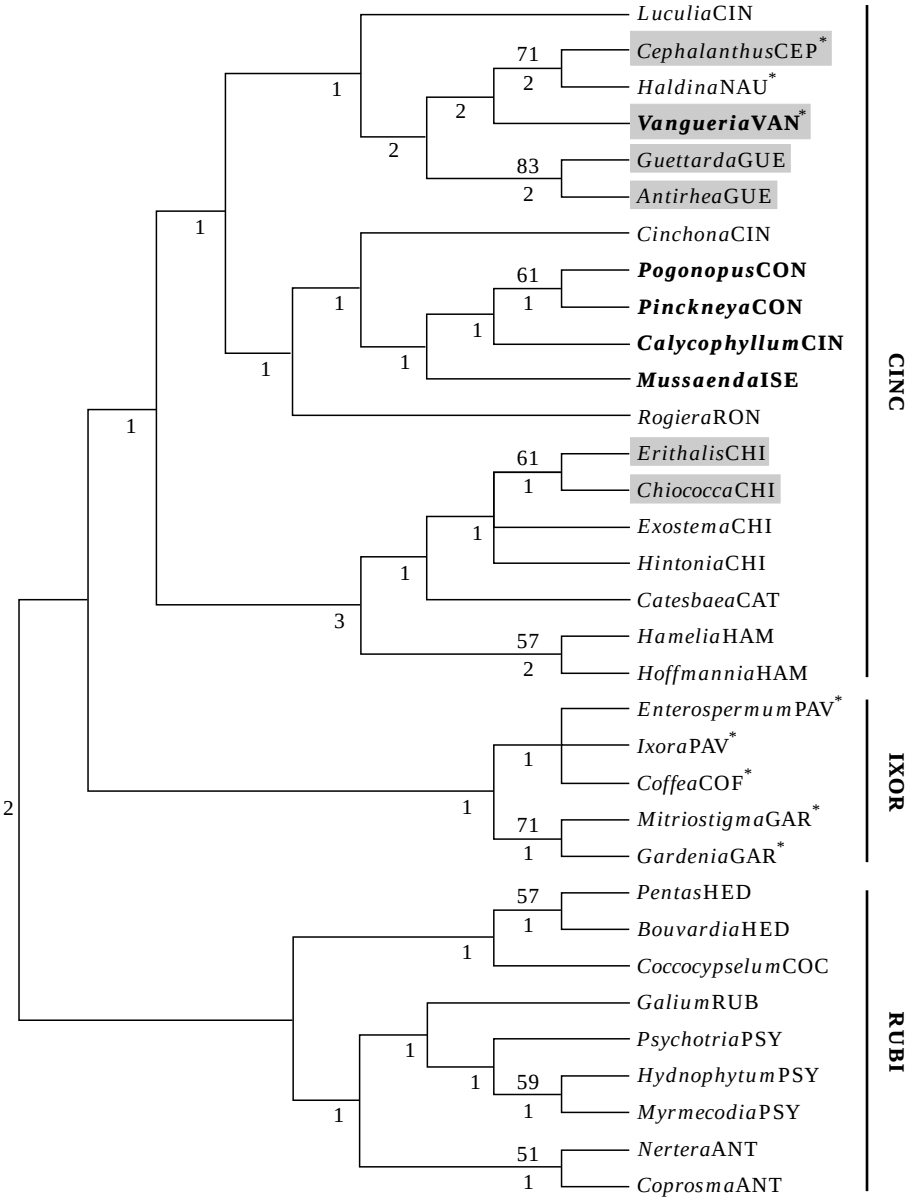

Fig. 1. Strict consensus tree of the 6 equally parsimonious trees of Rubiaceae based on morphological characters. The tribal positions are indicated by a three-letter suffix corresponding to the tribes in Table 1. Vertical bars and corresponding letters represent: CINC=subfamily Cinchonoideae, IXOR= subfamily Ixoroideae, RUBI=subfamily Rubioideae. Taxon names in boldface have different subfamilial position in the different analyses. Shaded genera are members of Robbrecht's (1988) subfamily Antirheoideae. Stars (*) indicate taxa with ixoroid pollen presentation mechanism. Numbers above nodes indicate bootstrap fractions above 50%. Numbers below branches are branch support values= number of extra steps required to collapse the particular node.

*Calycophyllum*CIN). In the molecular investigation, all these genera are connected to the base of the Ixoroideae taxa (Fig. 2, IXOR s.l.) but in the morphological analysis they are nested within the Cinchonoideae subfamily group (Fig. 1, CINC), more in agreement with the current classification.

The combined morphological and molecular analysis —(Fig. 4, Tables 2, 3) resulted in 6 equally parsimonious trees (S=1131, CI=0.447, RI=0.701, T=285, TI=0.252).

Table 2
Tree information

	n	nc	np	S	CI	RI	T	TI
Morphological analysis, multistate taxa as polymorphic	33	35	6	179	0.598	0.673	36	0.201
Morphological analysis, multistate taxa as uncertain	33	35	6	135	0.467	0.673	36	0.267
Molecular analysis	33	379	13	979	0.451	0.714	245	0.25
Combined analysis	33	414	6	1131	0.447	0.701	285	0.252
Combined matrix optimized on morphological trees	33	414	—	1260	0.401	0.64	—	—
Combined matrix optimized on molecular trees	33	414	—	1134	0.445	0.7	—	—

Abbreviation used in the table: CI=consistency index; n=number of taxa; nc=number of phylogenetically informative characters; np=number of most parsimonious trees; S=length of most parsimonious trees; RI=retention index; T=total support, the sum of all branch support values over the tree (Kallersjö et al., 1992); TI=total support index, T/S (K. Bremer, 1995).

Table 3
Branch support values (B) and corresponding bootstrap fractions (%) (Figures in boldface are discussed in "Evaluation of the trees and branches")

Morphological tree (Fig. 1)		Molecular tree (Fig. 2)		Combined tree (Fig. 4)	
B	%	B	%	B	%
3	**<50**	46	199	50	100
2	83	45	100	45	100
2	71	20	99	21	100
2	57	18	100	21	98
2	<50	17	100	18	100
1	71	12	97	15	99
1	61	11	95	13	100
1	61	10	99	13	99
1	59	10	99	12	93
1	57	10	93	11	97
1	51	8	99	10	99
1	<50	7	80	9	99
0	52	6	81	9	98
		5	91	7	83
		3	61	7	79
		2	**97**	5	73
		2	**74**	4	70
		2	**57**	2	68
		2	**<50**	2	56
		1	66	2	53
		1	56	2	51
		1	<50	1	67
		0	50	0	63
				0	63
				1	<50
				2	<50

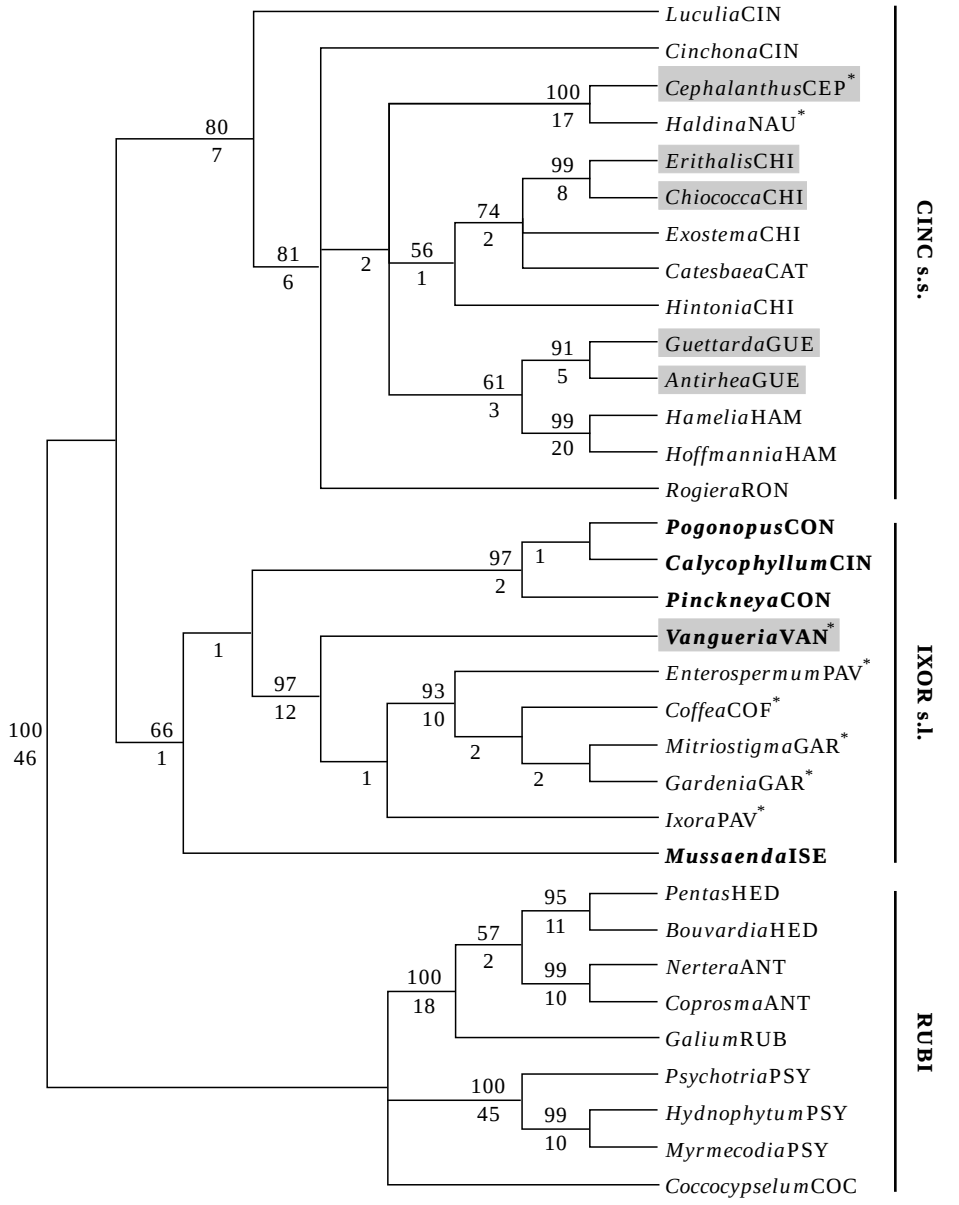

Fig. 2. Strict consensus tree of the 13 equally parsimonious trees of Rubiaceae based on cpDNA characters. The tribal positions are indicated by a three-letter suffix corresponding to the tribes in Table 1. Vertical bars and corresponding letters represent: CINC s.s.=subfamily Cinchonoideae s.s., IXOR s.l.=subfamily Ixoroideae s.l., RUBI=subfamily Rubioideae. Taxon names in boldface have different subfamilial position in the different analyses. Shaded genera are members of Robbrecht's (1988) subfamily Antirheoideae. Stars (*) indicate taxa with ixoroid pollen presentation mechanism. Numbers above nodes indicate bootstrap fractions above 50%. Numbers below branches are branch support values=number of extra steps required to collapse the particular node.

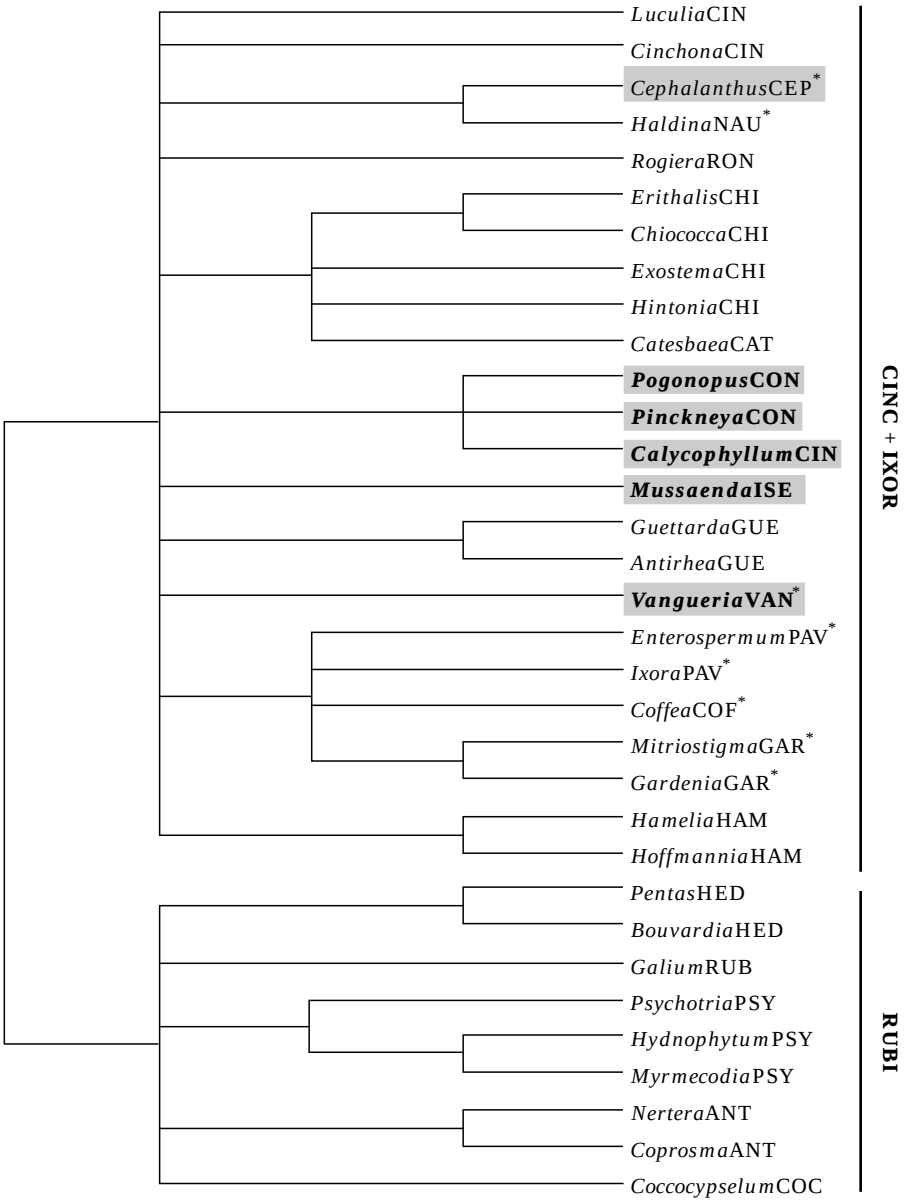

Fig. 3. Strict consensus tree of the 19 (6+13) morphological and cpDNA trees. The tribal positions are indicated by a three-letter suffix corresponding to the tribes in Table 1. Vertical bars and corresponding letters represent: CINC=subfamily Cinchonoideae, IXOR=subfamily Ixoroideae, RUBI= subfamily Rubioideae. Taxon names in boldface have different subfamilial position in the different analyses. Shaded genera are members of Robbrecht's (1988) subfamily Antirheoideae. Stars (*) indicate taxa with ixoroid pollen presentation mechanism.

The individual branch support (B) values vary between 1 and 50 steps. This tree requires a very high number (51) of extra steps to be collapsed. Twenty-two nodes

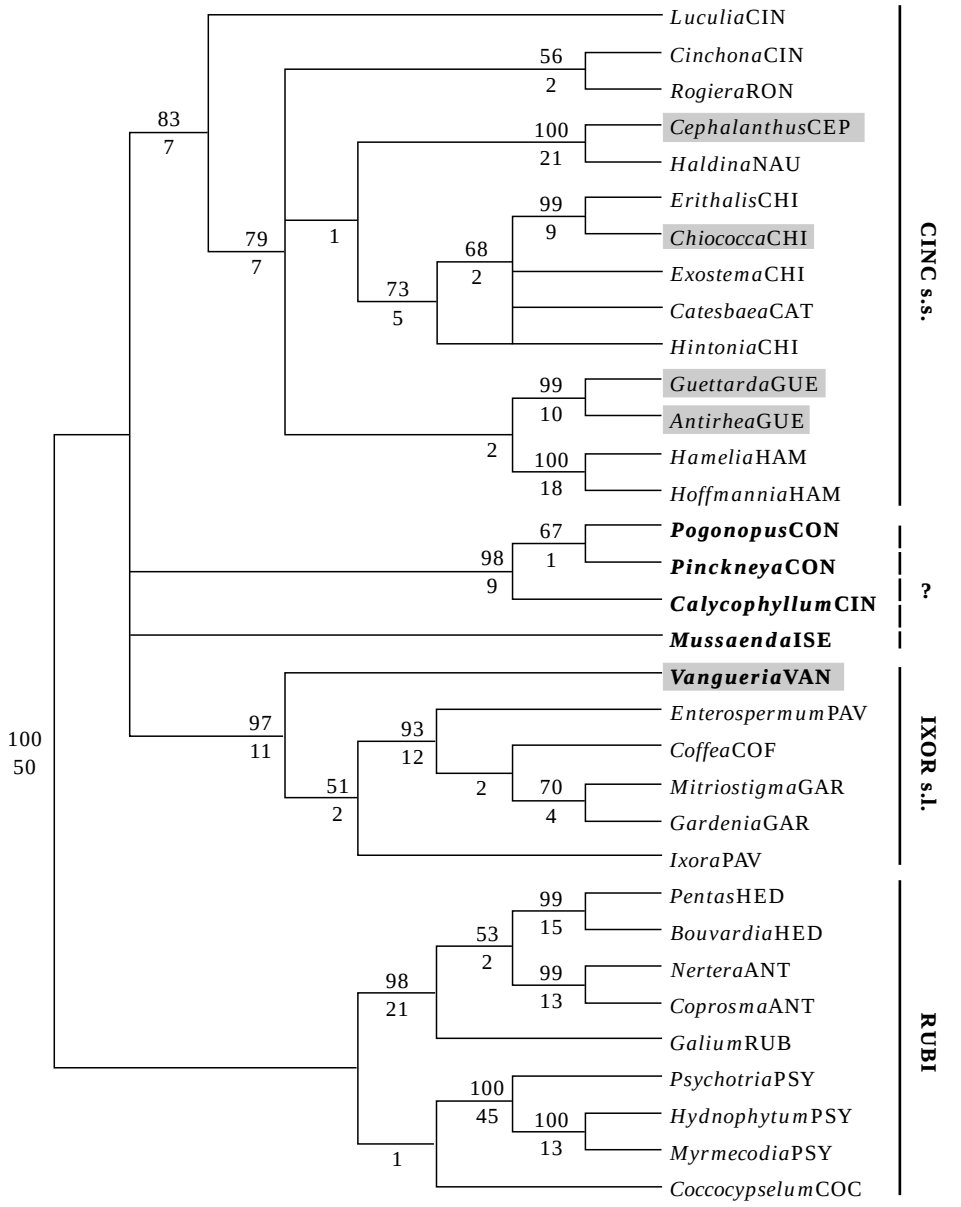

Fig. 4. Strict consensus tree of the 6 equally parsimonious trees of Rubiaceae based on a combined data set of morphological and cpDNA characters. The tribal positions are indicated by a three-letter suffix corresponding to the tribes in Table 1. Vertical bars and corresponding letters represent CINC s.s.=subfamily Cinchonoideae s.s., IXOR s.l.=subfamily Ixoroideae s.l., RUBI=subfamily Rubioideae. Taxon names in boldface have different subfamilial position in the different analyses. Shaded genera are members of Robbrecht's (1988) subfamily Antirheoideae. Stars (*) indicate taxa with ixoroid pollen presentation mechanism. Numbers above nodes indicate bootstrap fractions above 50%. Numbers below branches are branch support values=number of extra steps required to collapse the particular node.

have bootstrap fractions above 50%, 12 above 95%. The three subfamilies Rubioideae, Cinchonoideae and Ixoroideae are supported in this analysis as in the previous ones. The genus *Vangueria* is placed close to the Ixoroideae s.s., as in the DNA analysis, in contradiction to the morphological analysis. The position of the taxa with a semaphyll (*Mussaenda*ISE, *Pogonopus*CON, *Pinckneya*CON, *Calycophyllum*CIN) is unresolved in the consensus tree due to different equally parsimonious trees; some trees indicate a relationship to the base of the Ixoroideae as in the DNA analysis and other trees indicate a relationship to the Cinchonoideae as in the morphological analysis.

The combined matrix optimized on the morphological and molecular trees —(Table 2). When the combined matrix was optimized on trees from the morphological analysis, the length increased by 129 steps (S=1260, CI=0.401, RI=0.640). When the combined matrix was optimized on the molecular trees, the length increased by 3 steps (S=1123, CI=0.445, RI=0.700).

Discussion

Parsimony is a central concept in phylogenetic analysis and, as stated by Farris (1983: 36), "phylogenetic systematics alone provides a logical basis for the empirical study of the relationships among organisms" and "phylogenetic analysis is necessarily based on parsimony". With the parsimony criterion, we cannot accept consensus trees for classification or as phylogenetic hypotheses as these are not the most parsimonious solutions (Miyamoto, 1985). The parsimony principle is the only criterion needed for choosing a tree from one analysis instead of a consensus tree based on trees from analysis of one or more separate data sets. Kluge (1989: 10) stated that "total evidence" (Carnap, 1950; Hempel, 1965) "is an important maxim in phylogenetic systematics". It is, naturally, as in all sciences; all information should be considered, but it is irrelevant for the choice of trees (i.e. most parsimonious trees rather than consensus trees) for construction of phylogenetic hypotheses.

A few authors (e.g. Bull et al., 1993), have argued for not combining data sets if they are "incongruent" but as we do not know the true phylogeny we do not know which data set is "incorrect". The most parsimonious solution to all data must be a combined analysis, in the same way as we analyse incongruent characters from a single data set.

What does this particular case study of the Rubiaceae tell us about the differences between the trees from the separate analyses, the combined analysis and the consensus tree, and what effects do these differences have on classifications? The strict consensus tree of all trees from the two analyses (Fig. 3) is very collapsed with little explanatory power. It is virtually useless for classification, especially at higher levels, as only a few branches are resolved. The six trees from the combined analysis (Fig. 4) represent the most parsimonious solutions (1131 steps) for molecular and morphological data. In comparison with trees from the separate analyses, these trees are 129 steps shorter than the morphological trees with all characters optimized (1260 steps), or three steps shorter than the molecular trees (1134 steps with all characters optimized).

A systematic/phylogenetic study is not a static process where all characters are

investigated and analysed just once. The investigation is a dynamic process with alternating character studies and analyses. In many cases, morphological homologies are difficult to identify and can sometimes be understood only after a cladistic analysis. Such reinvestigations are frequently referred to as reciprocal illumination (Hennig, 1966). In other cases, conflicts between different analyses stimulate the investigator to carry out more detailed investigations of the characters and taxa involved.

Within Rubiaceae, the genus *Vangueria* is positioned in different subfamilies in the separate morphological and molecular analyses. One presumably important morphological character is the "ixoroid pollen presentation mechanism". This occurs in some representatives of the Cinchonoideae (CINC) and all members of Ixoroideae (IXOR) (Figs 1, 2). *Vangueria* (VAN) is nested within CINC in the morphological analysis (Fig. 1), and is placed basally in the IXOR clade in both the molecular and the combined analyses (Figs 2, 4). Detailed investigations of the pollen presentation mechanism have recently been performed. As predicted, different mechanisms and structures were discovered in the CINC and IXOR taxa. In *Ixora platythyrsa* (IXOR) the pollen is presented on the back of the adpressed stigma lobes and the lobes do not open until the next day (Nilsson et al., 1990); in *Cephalanthus occidentalis* (CINC) there are no lobes and it has been shown (Imbert and Richards, 1993) that the pollen is presented on "the soon-to-be-receptive stigmatic surface". To date, no member of the tribe Vanguerieae has been investigated in detail with respect to pollen presentation, but from the combined analysis it is predicted to have the IXOR type. Cladistic analyses clarify homologies or potential lack of homology.

Evaluation of the trees and branches —To evaluate stability of particular branches or whole trees, various methods are available (e.g. Felsenstein, 1985; Bremer, 1988, 1995; Sanderson, 1989; Kallersjø et al., 1992; Hillis and Bull, 1993). In this paper bootstrap fractions, branch support values (B), total support (T), and the total support index (TI) are calculated and compared between the different analyses (Figs 1, 2, 4, Table 2).

Bootstrap fractions, from 1000 replicates, are much higher and support many more branches in the molecular and the combined analysis than in the morphological analysis (Table 3). The same pattern emerges from the branch support values (Table 3). It is striking that the morphological analysis has very low branch support values (Fig. 1, Table 3); no branch has a higher value than 3 steps. The average branch support is 1.2 (T divided by the number of possible branches; the latter value equals 2n–3 where n is the number of taxa), while the molecular tree has branch support values up to 50 (average 9.3) (Fig. 2, Table 3). One of the reasons for these differences is the different number of characters between the analyses; the morphological analysis has only 35 informative characters while the molecular has 414. The branch support values (and total support) are positively correlated with the number of characters and they are thus not very useful if different analyses are compared; they are informative within an analysis as they indicate the relative stability of the particular branches.

Both bootstrap fractions and branch support values are used to evaluate the stability of particular nodes, and it is interesting to see how these values can differ from each other (Figs 1, 2, 4, Table 3). In the molecular tree, four different nodes,

all with a branch support value of 2 steps, have bootstrap fractions of 97, 74, 52, and below 50%, respectively. In the morphological tree, the node with the highest branch support value of 3 steps has bootstrap fraction below 50%. The reasons for these differences are unclear, but large differences are presumably more likely to occur on short branches and if the particular characters have low ci values.

When the whole tree stability is considered, the total support index (TI; K. Bremer, 1995) has been used; this index is not correlated to the number of characters. Interestingly, in this study the morphological analysis has the highest TI value among the analyses despite the low branch support values. This means that the morphological characters are more consistent among themselves in supporting a particular resolution than are the molecular characters. Fewer in number as they are, the morphological characters on the other hand provide less support to the individual branches than the more numerous molecular characters do.

Implication for Rubiaceae systematics —These results are preliminary in that only ca. 5% of the genera are investigated but the implications for several phylogenetic issues may be discussed. In all analyses the taxa are distributed in three groups more or less congruent to three of the four subfamilies (Robbrecht, 1988, 1993b). The molecular, morphological and combined consensus trees differ primarily in the placement of five taxa (boldface in figures). The results ought to convince the traditionally orientated taxonomists that phylogenetic analysis with cladistic methods will improve classification of the Rubiaceae.

The subfamily Rubioideae is circumscribed in exactly the same way in all analyses; the relationships within tribes are the same, although relationships among tribes differ. In all three analyses the tribe Hamelieae (HAM) is placed in CINC corroborating previous studies (Bremer and Jansen, 1991; Bremer and Struwe, 1992) and contradicting Robbrecht's (1993b) placement in the Rubioideae.

In all analyses two main branches corresponding to the subfamilies Ixoroideae and Cinchonoideae occur, but the boundary between these is so far not clear. There are four Cinchonoideae taxa (fide Robbrecht, 1988, 1993b) that are positioned either basally in Ixoroideae (RFLP/*rbc*L analyses, four of the six trees in the combined analysis, Figs 2, 4) or basally in Cinchonoideae (morphology, Fig. 2).

The wide circumscription of the fourth of Robbrecht's (1988, 1993b) subfamilies, the Antirheoideae, is contradicted by the results in all analyses and even by the very collapsed consensus tree (Fig. 3). There is definitely no support for the combination of Cephalantheae (*Cephalanthus*), Chiococceae (*Erithalis+Chiococca*), and Vanguerieae (*Vangueria*) with the Guettardeae (*Anthirhea+Guettarda*) into a large subfamily Antirheoideae, as these taxa are placed on four different branches. *Vangueria* and its position have been discussed above. *Cephalanthus* is definitely a member of the Naucleeae group as in Verdcourt's (1958) classification, supported by this and earlier studies (Bremer and Jansen, 1991; Bremer and Struwe, 1992; Bremer et al., 1995). In this and earlier studies (Bremer and Jansen, 1991; Bremer and Struwe, 1992), it has also been shown that Chiococceae s.s. are not close to the Guettardeae but closely related to a number of taxa earlier included in the tribes Cinchonoideae, Condamineae subtribe Portlandiinae and *Catesbaea* (in this study *Exostema, Hintonia* and *Catesbaea*), all characterized by unique morphological structures of the stamens: filaments fused into a basal ring and linear, mostly basifixed

anthers. In Bremer (1992), the tribe Chiococceae was widened to include all taxa with these and other characters (funnelform or rotate corollas, imbricate aestivation, mostly villous filaments, linear anthers and entire stigmas). However, Robbrecht (1993a,b) maintained the position of Chiococceae s.s. in the Antirheoideae and the other taxa in subfamily Cinchonoideae; he could not accept the results from the molecular studies because placentation of the Chiococceae s.s. differs from that of the others and he concluded "it is difficult to imagine a derivation from one placental type to the other" (Robbrecht, 1993a: 12).

The trees from the separate analyses differ distinctly from each other (as can be seen in the consensus tree). However, in comparison with current infrafamilial classification, circumscriptions of subfamilies and tribes, the results from the separate and the combined analyses do not differ very much. The only clear exception is the position of the genus *Vangueria* (discussed above).

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Appendix 1
Characters and character states used in the morphological analysis

Habit
1.[a] 0—Stems woody
1—Stems herbaceous[b]
2—*Stems with condensed internodes associated with epiphytic habit*
2. 0—Without distinct lenticels on stems
1—With distinct lenticels on stems
3. 0—Without myrmecophily
1—With myrmecophily
4. 0—External indumentum not of articulate type[c]
1—External indumentum of articulate type
2—*Glabrous*
3—*External indumentum with a Ca-oxalate crystals in hair lumen*
5. 0—Plants bisexual
1—Plants unisexual

Leaves
9. 0—Leaves without "moiré" striation pattern
1—Leaves with "moiré" striation pattern

Inflorescences and flowers
10. 0—Inflorescences axillary
1—Inflorescences terminal
11. 0—Flowers not in dense heads
1—Flowers in dense heads
12. 0—Without club-shaped bracts between the flowers
1—With club-shaped bracts between the flowers
14. 0—Ixoroid pollen mechanism absent
1—Ixoroid pollen mechanism present[d]
15. 0—Without semaphylls
1—With semaphylls[e]

Corolla
16. 0—Aestivation valvate
1—Aestivation contorted to the right
2—Aestivation imbricate *or subimbricate at apex*

3—Aestivation contorted to the left
17. 0—Corolla rotate
1—Corolla salver-shaped
2—Corolla funnel-shaped
3—Corolla campanulate
4—*Corolla tubular or narrowly funnel-shaped*
18. 0—Inside of corolla glabrous or irregularly hairy
1—Upper part of corolla hairy
2—Lower part of corolla hairy
3—*Upper part of corolla density hairy in connection with faucial ring*
4—*Inside of corolla distinctly hairy*

Stamens

19. 0—Stamens inserted at the base of the corolla
1—Stamens inserted near the mouth of the corolla
2—Stamens inserted at least one-fifth down from the mouth of the corolla, but not at the base
20. 0—Stamens not fused into a basal ring
1—Stamens fused into a basal ring[f]
21. 0—Filaments glabrous
1—Filaments hairy
48. 0—*Anthers dorsifix around middle*
1—*Anthers dorsifix very near the base*
2—*Anthers basifix*
53. 0—*Pollen grains in monades*
1—*Pollen grains in tetrades*
54. 0—*Pollen with 3 apertures*
1—*Pollen with >3 apertures*
55. 0—*Pollen colpate*
1—*Pollen colporate*
2—*Pollen porate*

Pistil

26. 0—Style glabrous
1—Style hairy
27. 0—Stigma entire, *clavate to scarcely widened*
1—Stigma lobate
2—*Stigma entire, cylindric to capitate or spindle-shaped*
29. 0—Stigma glabrous *or with very short hairs*
1—Stigma *distinctly* hairy
49. 0—*Ovary 2-locular*
1—*Ovary 1-locular*
2—*Ovary≥3-locular*

Fruit

30. 0—Drupe *with unilocular stones*
1—Berry
2—Capsule
3—*Dry indehiscent fruit*
4—*Drupe with plurilocular stones*
31. 0—Without lenticels on the fruits
1—With lenticels on the fruits
51. 0—*Placenta ±elongate, adnate to septum*
1—*Placenta ±stalked, attached to apical part of septum*
2—*Placenta ±stalked, attached to central part of septum*
4—*Placenta reduced or partly included inside the seed, ovules attached apically*
5—*Placenta reduced or partly included inside the seed, ovules attached centrally*
6—*Placenta reduced or partly included inside the seed, ovules attached basally*
7—*Placenta pulpy*
8—*Placenta not stalked but only a small part of placenta attached to septum*
9—*Placenta enlarged, adnate to septum*

Seeds

33. 0—One seed per carpel
1—Seeds numerous in each carpel
36. 0—Testa cells smooth
2—Testa cells with ridges, i.e., very large pits
3—Testa cells granulate to tuberculate
4—*Testa cells with fingerlike projections*

37. 0—Embryo at most one-fourth of the size of the endosperm
1—Embryo at least one-fourth of the size of the endosperm

Chemistry

42. 0—Complex indole-alkaloids absent
1—Complex indole-alkaloids present
43. 0—*Asperuloside present*[g]
1—*Asperuloside absent*
56. 0—*No accumulation of aluminum*[h]
1—*Accumulation of aluminum*

Chromosome number[i]

57. 0—*Chromosome number x=11*
1—*Chromosome number x=9*
2—Chromosome number x=10
3—*Chromosome number x=12*
4—*Chromosome number x=14*
5—*Chromosome number x=17*
6—*Chromosome number x=18*
7—*Chromosome number x=13*

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[a]The character numbers correspond to those in the data matrix (Appendix 2), they also correspond to the numbers of Bremer and Struwe (1992). Most characters or states are the same as in Bremer and Struwe (1992), however, new characters or states are included in this analysis and are in italics. Some numbers or states are missing in this appendix as they are not included in this particular analysis or in the Rubiaceae analysis in Bremer and Struwe (1992).

[b]Also plants with a basal woodiness are included.

[c]Cf. Robbrecht (1988).

[d]Cf. Bremekamp (1966).

[e]Cf. Leppik, (1956, 1977).

[f]Cf. Bremer (1992).

[g]Cf. Kooiman (1969).

[h]Cf. Chenery (1948).

[i]Cf. Fagerlind (1937), Kiehn (1985, 1986) and Kiehn (pers. comm.)

Appendix 2
Data matrix of morphological characters

Genus	Character number																																		
	1	2	3	4	5	9	11	12	14	15	16	17	18	19	20	21	26	27	29	30	31	33	36	37	42	43	44	48	49	51	53	54	55	56	57
*Luculia*CIN	0	1	0	0	0	0	0	0	0	0	2	1	0	1	0	0	0	1	0	2	0	1	2	0	—	—	0	0	0	0	0	0	1	0	0
*Cinchona*CIN	0	0	0	0	0	0	0	0	0	0	0	1	1	2	0	0	0	1	0	2	0	1	2	0	1	1	0	1	0	0	0	0	1	1	5
*Cephalanthus*CEP	0	1	0	0	0	1	1	1	1	0	2	2	0	1	0	0	0	2	0	3	0	0	2	1	1	1	0	1	0	—	0	0	1	0	0
*Haldina*NAU	0	1	0	0	0	1	1	1	1	0	2	2	0	1	0	0	0	2	0	2	0	1	2	0	1	—	0	2	0	1	0	0	1	0	0
*Rogiera*RON	0	0	0	0	0	0	0	0	0	0	2	1	1,3	1	0	0	0	1	0	2	0	1	2	0	1	1	0	0	0	2	0	0	1	0	0,2
*Erithalis*CHI	0	0	0	0	0	0	0	0	0	0	2	0	0	—	1	1	0	0	0	0	0	0	0	0	—	—	0	2	2	4	0	—	—	0	—
*Chiococca*CHI	0	0	0	0	0	0	0	0	0	0	2	2	0	0	1	1	0	0	0	0	0	0	2	0	—	1	0	2	0	4	0	0	1	0	0,3,4,7
*Exostema*CHI	0	0,1	0	0	0	0	0	0	0	0	2	4	0	0	1	0,1	0	0	0	2	0,1	1	0,2	0	1	—	0	2	0	8	0	0	1	0	7
*Hintonia*CHI	0	1	0	0	0	0	0	0	0	0	2	2	0	0	1	1	0	0	0	2	1	1	0	0	1	—	0	2	0	8	0	0	1	0	—
*Pogonopus*CON	0	1	0	0	0	0	0	0	0	1	0	4	2	2	0	0	0	1	0	2	1	1	0	1	—	—	0	0	0	0	0	0	1	0	—
*Pinckneya*CON	0	1	0	0	0	0	0	0	0	1	0	2	2	2	0	0	0	1	0	2	1	1	2	0	—	—	0	0	0	0	0	0	1	0	—
*Calycophyllum*CIN	0	1	0	0	0	0	0	0	0	1	2	2,3	2	—	0	0	0	1	0	2	0	1	2	0	—	—	0	0	0	0	0	0	1	0	5,6
*Mussaenda*lSE	0	1	0	0	0	0	0	0	0	1	0	2	1,4	2	0	0	0	1	0	1	0	1	2	0	0	1	0	1	0,2	9	0	1	1	0	0
*Guettarda*GUE	0	1	0	3	0,1	1	0	0	0	0	2	1,2	0	1	0	0	0	0	0	4	0	0	0	1	1	1	0	0	0,2	4	0	—	2	0	0
*Antirhea*GUE	0	1	0	3	1	1	0	0	0	0	2	1	0	1	0	0	0	1	0	4	0	0	2	1	1	—	0	0	0	—	0	0	2	0	—
*Vangueria*VAN	0	1	0	0	0	0	0	0	1	0	0	2	1	1	0	0	0	2	0	0	0	0	2	1	0	1	0	1	2	4	0	0	2	0	0
*Enterospermum*PAV	0	0	0	2	0	0	0	0	1	0	3	2	1	1	0	0	1	1	0	0	0	1	0	0	0	1	0	1	0,1	1	0	0	1	—	0
*Ixora*PAV	0	0	0	0	0	0	0	0	1	0	3	1	0	1	0	0	0,1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0
*Coffea*COF	0	0	0	2	0	0	0	0	1	0	3	2	0	1	0	0	0	1	0	0	0	0	0	0	—	1	0	0	0	5	0	0,1	1	0	0
*Mitriostigma*GAR	0	0	0	0	0	0	0	0	1	0	3	2,3	0	1	0	0	0	1	0	1	0	1	0	0	—	—	0	1	0	7	1	0	2	0	0
*Catesbaea*CAT	0	0	0	2	0	0	0	0	0	0	2	2	0	0	1	0	0	0	0	1	0	1	0	0	—	1	0	2	0	—	0	0	0	0	3
*Gardenia*GAR	0	0	0	2	0	0	0	0	1	0	3	2	0	1	0	0	0,1	0,1	0	1	0	1	2	0	0	1	0	0	1	7	1	0	2	0	0
*Hamelia*HAM	0	0	0	0	0	0	0	0	0	0	2	2,4	0	0	0	0	0	0,1	0	1	0	1	3	0	1	1	1	2	2	0	0	0	0	0	3
*Hoffmannia*HAM	0,1	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0,1	0	1	0	1	3	0	—	1	1	1	0	0,2	0	0	1	0	3
*Pentas*HED	1	0	0	1	0	0	0	0	0	0	0	2	1	1	0	0	0	1	0	2	0	1	0	0	0	0	1	1	0	2	0	0,3	1	0	2
*Bouvardia*HED	1	0	0	1	0	0	0	0	0	0	0	2,4	1	1	0	0	0	1	0	2	0	1	0	0	0	1	1	0	0	2	0	0	1	0	1
*Galium*RUB	1	0	0	0	0	0	0	0	0	0	0	0,2	0	—	0	0	0	1	0	0,3	0	0	—	0	0	0	1	0	0	6	0	1	1	0	0
*Psychotria*PSY	0,1	0,1	0	1	0	0	0	0	0	0	0	2	2	1,2	0	0	0	1	0	0	0	0	0	0	—	0	1	0	0	6	0	0,1	0,1,2	1	0
*Hydnophytum*PSY	2	0	1	2	0	0	0	0	0	0	0	1,2	2	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0,2	6	0	1	0	1	0
*Myrmecodia*PSY	2	0	1	2	0	0	0	0	0	0	0	2	2	1	0	0	0	1	0	0	0	0	0	0	—	1	1	0	0,3	6	0	0,1	1,2	0	0
*Nertera*ANT	1	0	0	0	1	0	0	0	0	0	0	2	0	2	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	6	0	0	1	0	0
*Coprosma*ANT	0	0	0	2	1	0	0	0	0	0	0	2	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	2	0	6	0	0	1	1	0
*Coccocypselum*COC	1	0	0	0	0	0	0	0	0	0	0	2	2	1,2	0	0	0	1	0	1	0	1	4	0	0	0	1	0,1	0	2	0	0	1	1	2

Species names are followed by a three-letter suffix corresponding to the three letters of the tribal names: ANThospermeae, CATesbaeeae, CEPhalantheae, CINchoneae, CHIococceae, COFfeeae, CONdamineeae, GARdenieae, GUEttardeae, HAMelieae, HEDyotideae, ISErtieae, MORindeae, NAUcleeae, PAVetteae, PSYchotrieae, RONdeletieae, RUBieae, VANguerieae.